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Detection of Influenza C Virus by Using an In Situ Esterase Assay

PAMELA C. WAGAMAN, H. ADELE SPENCE, AND RICHARD J. O'CALLAGHAN*

Department of Microbiology, Immunology, and Parasitology, School of Medicine, Louisiana State University Medical Center, New Orleans, Louisiana 70112

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A variety of chemically defined compounds were tested to characterize the substrate specificity of the influenza C virus esterase and to determine whether a substrate could be found that would be useful in an assay to detect the virus. Two new substrates, α-naphthyl acetate and α-naphthyl propionate, were identified; α-naphthyl acetate was employed to develop an assay specific for influenza type C virus in MDCK cells. The assay was sufficiently sensitive to detect esterase activity in a single cell and distinguished influenza C virus infections from those of types A and B viruses. Infected cells could be detected as early as 8 h postinfection, with maximal enzyme detection occurring at 24 h. Assay of influenza C virus in the chorioallantoic or amniotic fluid of infected eggs was performed by applying fluids directly onto nitrocellulose strips and then incubating with α-naphthyl acetate. Both the cellular and nitrocellulose-bound assays are rapid, inexpensive, and easy to perform, offering advantages for use in clinical laboratories.

Influenza type C virus usually causes mild upper respiratory infections, especially in young children (4, 12, 14). However, the virus may cause more severe infections such as bronchiolitis or pneumonia (23) or infections clinically indistinguishable from those caused by influenza type A virus (3). Serological studies indicate that influenza C infections occur worldwide (1, 6, 9–11, 18). Primary infections occur in early childhood, with an increase in numbers of seropositive children over 6 months of age. By 7 years of age, 80 to 95% of children tested are seropositive (3, 9). Infections occur in adults despite titers of antibody specific for the virus (12, 14). Hornsleth et al. have reported that influenza C virus infections in the elderly may occur concurrently with influenza A or B virus infections (10).

Influenza type C virus differs significantly in its growth requirements from type A and B viruses and as a result is seldom isolated and identified (5). The type C virus is best propagated in Madin-Darby canine kidney (MDCK) cell monolayers (17) or in 8- to 9-day-old embryonated hen eggs. The optimum temperature for cultivation is 33°C, and fluids from tissue culture or eggs may be assayed by hemagglutination. The hemagglutination assay is performed at 0 to 4°C to limit the receptor-destroying activity of the viral elution enzyme (5).

The enzyme of influenza C virus is located on the hemagglutinin molecule, a surface glycoprotein which also mediates attachment and penetration (16, 22). The viral enzyme is a 9-*O*-acetyl esterase, which hydrolyzes acetic acid from the viral receptor, 9-*O*-acetyl-*N*-acetylneuraminic acid, allowing release of the mature virus from infected cells or from erythrocytes (7, 8, 20). Vlasak et al. (22) have shown that *para*-nitrophenyl acetate (PNPA) may serve as a substrate for the viral enzyme in vitro.

Our study of the influenza C virus esterase led to the identification of new substrates and to the development of an in situ esterase assay that may have application in the diagnostic laboratory for the rapid and specific detection of influenza C virus.

* Corresponding author.

MATERIALS AND METHODS

Virus. Influenza C virus strain JJ (14) was propagated by inoculation of the chorioallantoic cavity of 9-day-old chicken embryos (certified pathogen-free eggs; SPAFAS, Inc., Norwich, Conn.) as described previously (19). After incubation for 3 days at 35°C, chorioallantoic fluid (CAF) was harvested, assayed by hemagglutination with chicken erythrocytes as previously described (21), and frozen at −70°C. CAF used for stock virus routinely contained approximately 256 hemagglutinin units per 0.1 ml. Influenza virus strains C/Cal (obtained from P. Palese, Mount Sinai School of Medicine, New York, N.Y.) and C/JHB (obtained from R. Compans, University of Alabama Medical Center, Birmingham) were propagated by amniotic inoculation as described previously (19). Influenza virus strains A/PR8 and B/Lee were cultivated in the amniotic cavity of 11-day chicken embryos at 35°C for 3 days. Mumps virus was obtained from C. Howe, Louisiana State University Medical Center, New Orleans. Parainfluenza virus type 3 and respiratory syncytial viruses were obtained from R. Gohd, Charity Hospital, New Orleans, La.

Analysis of esterase substrates. α-Naphthyl acetate (ANA; 5 mM) and α-naphthyl propionate (ANP; 5 mM) (Sigma Chemical Co., St. Louis, Mo.) were prepared by the method of Li et al. (13) by dissolving ANA (10 mg) or ANP (10 mg) in ethylene glycol monomethyl ether (0.5 to 1.0 ml) and diluting to 10 ml with phosphate buffer (0.067 M, pH 6.3 or 7.4). Hexazonium pararosanilin was prepared as previously described by Yam et al. (24).

PNPA (1 mM), a chromogenic esterase substrate, was prepared by dissolving PNPA (18 mg) in 95% ethanol (5 ml) and diluting with phosphate buffer (0.067 M, 95 ml, pH 6.3 or 7.4).

To analyze hydrolysis of substrates by virions, CAF from influenza C/JJ virus-infected or normal eggs (50 μl) was equilibrated to a temperature of 23, 32, 37, or 50°C. Prewarmed substrate solution (50 μl, pH 6.3 or 7.4) was added, and tubes were incubated for 15 min. Substrate alone was observed at each temperature as a control for autohydrolysis. Positive reactions were determined by observing the relative amount of garnet-colored insoluble product formed

TABLE 1. Analysis of substrates for influenza C virus[a]

pH	Temp (°C)	ANA[b]			ANP[b]			Hydrolysis of PNPA[c]		
		CAF		Substrate control[d]	CAF		Substrate control	CAF		Substrate control
		Normal	Infected		Normal	Infected		Normal	Infected	
6.3	23	±	++++	−	+	++	−	0.25	0.88	0.07
	33	+	+++	−	+	+	−	0.35	1.00	0.01
	37	±	++	−	±	+	−	0.35	1.07	0.02
	50	−	±	−	−	−	−	0.63	1.23	0.06
7.4	23	+	+++	−	±	+	−	0.57	2.01	0.10
	33	+	++	−	±	±	−	0.90	1.71	0.46
	37	±	+	−	±	−	−	1.23	2.06	0.75
	50	−	±	+	−	−	−	2.12	2.29	1.90

[a] Substrate solutions, stock influenza C/JJ virus, and normal CAF were preheated to 23, 33, 37, or 50°C. Substrate (50 μl) and virus-infected CAF (50 μl) or normal CAF (50 μl) were mixed and incubated for 15 min before observing for insoluble product (ANA or ANP) or determining the optical density at 405 nm (PNPA).

[b] ±, faint, rose-colored insoluble reaction product; −, negative reaction with no evidence of garnet-colored insoluble product; + to ++++, increasing intensity of garnet-colored insoluble reaction product.

[c] Hydrolysis of PNPA was determined by optical density at 405 nm. Values reported are averages of three separate determinations.

[d] For a substrate control, 100 μl of substrate reaction solution was incubated without added virus or normal CAF to determine the extent of autohydrolysis.

upon hydrolysis of ANA or ANP (− to ++++) or the change in optical density at 405 nm for PNPA hydrolysis.

Cells. The MDCK cells used in these experiments were shown by culture to be free of mycoplasmas. Monolayers were established in 35- by 10-mm tissue culture dishes (Costar, Cambridge, Mass.) or 6-well tissue culture plates (Corning Glass Works, Corning, N.Y.) by cultivation of approximately 3×10^5 MDCK cells, in 2 ml of Earle minimal essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum, per well. Monolayers were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 5 to 7 days. Primary monkey kidney cells were obtained from Viromed Laboratories, Inc., Minnetonka, Minn.

Infection of monolayers with virus. Stock influenza C/JJ virus was diluted in sterile phosphate-buffered saline (10 mM, pH 7.1) containing 0.2% bovine serum albumin. Confluent monolayers of MDCK cells were washed in phosphate-buffered saline–bovine serum albumin, inoculated with 0.2 ml of virus, and incubated for 1 h at 33°C in an atmosphere of 5% CO_2 to allow attachment of virus particles. The inoculum was removed, the cells were rinsed in phosphate-buffered saline–bovine serum albumin, and MEM containing 10% fetal calf serum was added to monolayers. Plates were incubated at 33 or 37°C in a humidified atmosphere with 5% CO_2 for 24 to 72 h. For plaque assays, infected monolayers were covered with 2 ml of 0.6% agarose overlay (Sea Plaque Agarose; FMC Bio Products, Rockland, Maine) prepared in MEM containing glutamine (1%), glucose (0.1%), penicillin (200 U/ml), streptomycin (200 μg/ml), trypsin (0.5 μg/ml), and DEAE-dextran (100 μg/ml). Monolayers were incubated for 72 h before removal of the overlay and detection of plaques or esterase activity.

In situ esterase detection. The method of Li et al. (14) was modified to detect enzyme activity in monolayers. After removal of the overlay or MEM, monolayers were fixed for 30 s with cold buffered Formalin-acetone (1.4 mM Na_2HPO_4, 7.3 mM KH_2PO_4, 45% acetone, 25% Formalin) and washed three times with distilled water. The fixed cells were incubated with 1 to 2 ml of ANA-pararosanilin solution (ANA-P) per well (35 mm) for 15 min at room temperature or 37°C.

Detection of plaques. After removal of the agarose overlay, monolayers were fixed for 1 min with absolute methanol at 0 to 4 C and stained with 1% aqueous crystal violet diluted 1:2 with absolute methanol. Alternatively, plates that had been fixed and tested for esterase activity were photographed and then stained with the crystal violet.

Neutralization studies. Stock influenza C/JJ virus was diluted 1:10 in phosphate-buffered saline–bovine serum albumin containing 10% serum from unimmunized rabbits or from rabbits immunized with influenza C virus. After incubation at 37°C for 60 min, the mixture (0.2 ml) was inoculated onto MDCK monolayers. After time was allowed for viral attachment, the monolayers were incubated and assayed for esterase activity or plaque formation.

Esterase detection in egg fluids. To screen egg fluids for esterase activity, CAF (10 μl) from eggs infected with influenza C/JJ virus or amniotic fluid from eggs infected with influenza A/PR8, B/Lee, C/Cal, or C/JHB virus were pipetted onto strips of nitrocellulose (5 by 10 cm; Schleicher & Schuell Co., Keene, N.H.). Uninfected CAF and amniotic fluid were included as negative controls. Strips of nitrocellulose were immersed in ANA-P and incubated for 15 min at room temperature before the reaction was read.

RESULTS

Analysis of esterase substrates. Influenza C virus was incubated with three different esters to determine the reactivity of these compounds with the viral esterase. The esters tested were ANA, ANP, and, as a positive control, PNPA (Table 1). CAF from uninfected eggs served as a negative control, and autohydrolysis of substrate was monitored by incubation of the substrate solution without CAF or virus.

ANA was hydrolyzed by CAF from infected eggs at 23 and 33°C at both pH 6.3 and 7.4. Minimal ANA hydrolysis was produced by normal CAF at the temperatures and pHs tested. ANA did not undergo autohydrolysis at either pH or at any of the temperatures tested. ANA at pH 6.3 was distinctly more reactive with virus-infected CAF than were the other substrates tested and was chosen for use in subsequent assays.

ANP did not undergo autohydrolysis under the conditions employed. ANP was hydrolyzed by virus-infected CAF to an extent consistently greater than that achieved by normal CAF.

PNPA, although hydrolyzed rapidly by CAF from virus-infected eggs only, was not chosen for analysis of infected

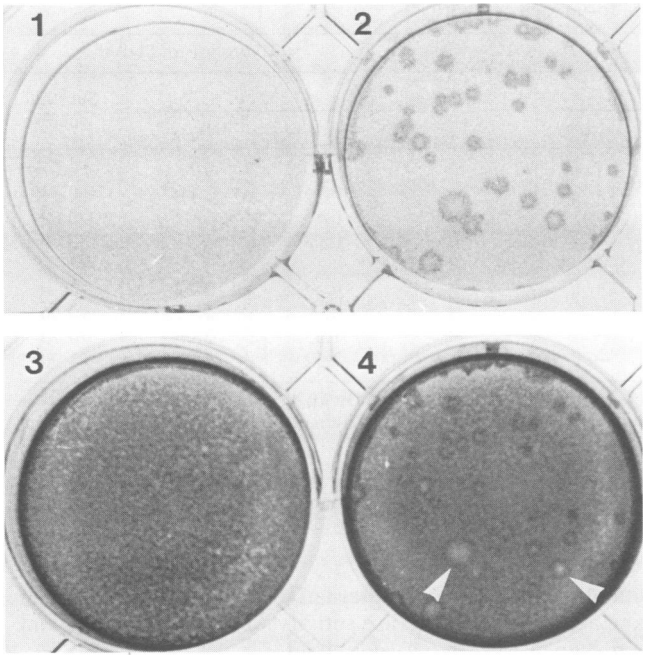

FIG. 1. Esterase activity and plaque production of influenza C/JJ virus. Confluent monolayers of MDCK cells were infected with a 10^{-5} dilution of stock influenza C/JJ virus (wells 2 and 4) or with normal CAF (wells 1 and 3). Plates were incubated with an agarose-trypsin overlay at 33°C. Monolayers were fixed with cold buffered Formalin-acetone and allowed to react with ANA-P to show esterase activity (wells 1 and 2) and photographed. The same plate was stained with crystal violet to show plaques (wells 3 and 4). Note the congruence of esterase-positive foci (well 2) with plaque formation (well 4). In well 4, several esterase-positive foci are seen without evidence of destroyed cells. Arrows show plaques outlined by esterase-positive cells.

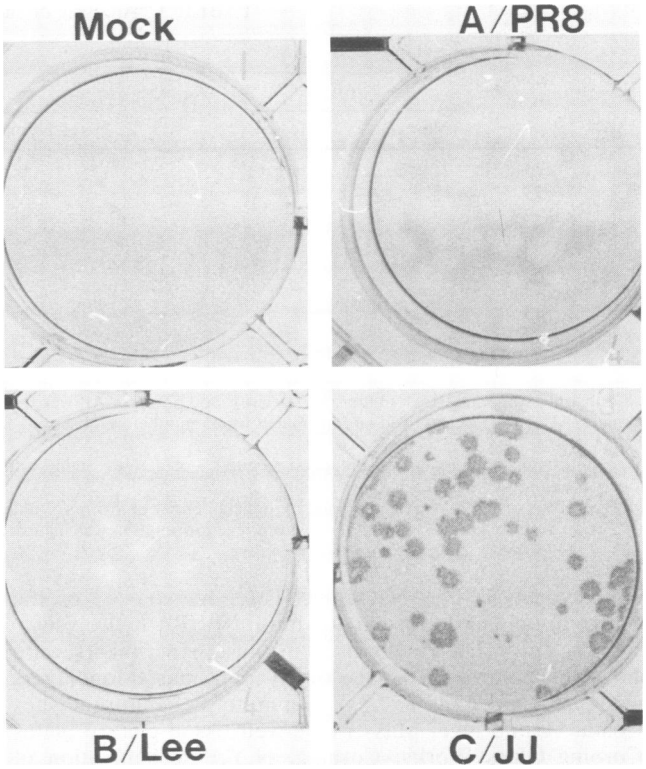

FIG. 2. Lack of esterase activity in MDCK monolayers infected with influenza A/PR8 or B/Lee virus. Confluent monolayers of MDCK cells were mock infected (well 1) or infected with a 10^{-4} dilution of stock influenza type A/PR8 (well 2) or type B/Lee (well 3) virus. Monolayers were incubated at 37°C with an agarose-trypsin overlay. Fixed monolayers were incubated with ANA-P to test for esterase activity. Monolayers infected with influenza type C virus as described in the legend Fig. 1 are included for comparison. Note the production of plaques by influenza type A and B virus in the absence of esterase activity (wells 2 and 3).

monolayers because it undergoes autohydrolysis, especially at slightly alkaline pHs.

Esterase assay of uninfected cell lines. To determine whether ANA-P could be used to identify influenza C virus-infected cells, uninfected primary monkey kidney cells and MDCK cells were tested for endogenous esterase activity. Monolayers of monkey kidney cells, when incubated with ANA-P at room temperature, hydrolyzed ANA. When tested under similar conditions, monolayers of MDCK cells failed to hydrolyze ANA.

Specificity of assay for influenza type C virus. To develop an in situ esterase detection assay specific for influenza C virus in MDCK cells, an agarose overlay was used to generate foci of infected cells, allowing maximal differentiation of infected cells from surrounding uninfected cells. Confluent MDCK cell monolayers were infected with influenza A/PR8, B/Lee, or C/JJ virus and incubated for 72 h at 33°C under an agarose overlay. Monolayers infected with influenza C virus showed distinct foci of esterase activity when tested with ANA-P (Fig. 1, well 2). No esterase activity could be demonstrated in mock-infected cells (Fig. 1, well 1). Cells infected with influenza A or B viruses at 33°C lacked esterase activity (data not shown). The esterase-positive foci in influenza C virus-infected monolayers contained garnet-colored cells and could be detected macroscopically as early as 24 h postinfection. By counterstaining the same plate with crystal violet to detect plaques, lytic plaques were shown within esterase positive foci, i.e., plaques and foci were congruent (Fig. 1, well 4). More foci than lytic plaques were evident in these plates, indicating that the esterase assay detected areas of the monolayer that were infected but were free of detectable cell lysis.

Another measure of the specificity of the esterase assay was obtained by neutralization of influenza C virus with specific rabbit antiserum. The antiserum inhibited infectivity and thus prevented esterase activity. Cells infected with virus that had been preincubated with normal rabbit serum failed to show a reduction in either esterase activity or plaque production.

As a further demonstration of the specificity of the esterase assay for influenza C virus, MDCK cells were infected with type C virus and incubated at 37°C. Influenza C virus replication is inhibited at 37°C; as expected, infected monolayers showed neither esterase activity nor viral plaques. This lack of esterase activity was not due to the inhibition of esterase activity at 37°C, because the influenza C virus esterase of whole virus hydrolyzed ANA at 37°C. Furthermore, MDCK cells infected with influenza C virus at 33°C possessed esterase activity when assayed at 37°C.

Influenza A and B viruses showed no evidence of esterase activity at 37°C (Fig. 2). Holes were evident in the cell monolayer, and crystal violet counterstaining of the same plates confirmed that these were viral plaques.

Esterase activity in monolayers cultured without trypsin.

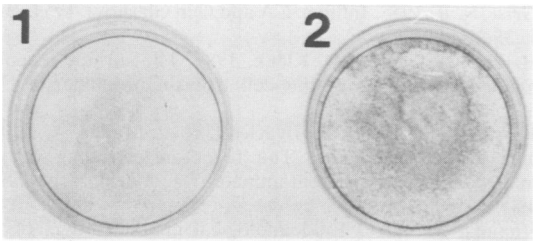

FIG. 3. Detection of influenza C virus in monolayers incubated with liquid medium. Confluent MDCK monolayers were infected with influenza C/JJ virus and incubated with MEM (supplemented with 10% fetal calf serum) without trypsin. Monolayers were fixed with cold buffered Formalin-acetone and incubated with ANA-P for detection of esterase activity. Wells: 1, mock-infected cells; 2, MDCK cells infected with C/JJ virus, 24 h postinfection.

The esterase assay was also tested for its utility in detecting influenza C virus-infected cells in monolayers incubated with liquid medium (MEM), i.e., without an agarose overlay or added trypsin. MDCK monolayers infected with influenza C virus and incubated with MEM at 33°C showed strong reactivity with ANA-P, demonstrated by widespread distribution of garnet-colored cells (Fig. 3, well 2). Esterase activity was maximal at 24 h, and reduced amounts of activity were detected microscopically as early as 8 h postinfection and as late as 72 h postinfection. No esterase activity was detected in monolayers infected with influenza A or B viruses and incubated at 33 or 37°C.

Application of the assay for screening egg fluids. ANA-P was also tested for its utility in screening infected egg fluids for the presence of influenza C virus. Samples of infected or uninfected egg fluids were pipetted onto nitrocellulose strips and incubated with ANA-P. CAF from uninfected eggs (Fig. 4, spot 1) and amniotic fluid (data not shown), as well as amniotic fluid of embryos infected with influenza A/PR8 (Fig. 4, spot 2) or B/Lee (Fig. 4, spot 3) virus, showed negligible reactivity with ANA-P. Three strains of influenza C (JJ, Cal, and JHB) showed distinct reactivity when incubated with ANA-P (Fig. 4, spots 4 to 6). Analysis of serial dilutions of ANA-positive fluids by using the nitrocellulose assay showed an apparent dose response of enzyme reactivity to viral concentration. In addition, respiratory syncytial virus (three different isolates), parainfluenza type 3 virus, and mumps virus were similarly tested on nitrocellulose strips. All isolates lacked reactivity with ANA-P.

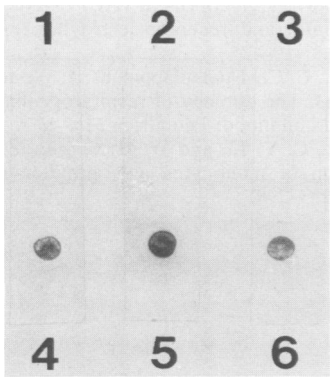

FIG. 4. Detection of influenza C virus in egg fluids. Normal CAF (spot 1), CAF from eggs infected with influenza C/JJ virus (spot 4), or amniotic fluid from eggs infected with influenza A/PR8 (spot 2), B/Lee (spot 3), C/Cal (spot 5), or C/JHB (spot 6) virus were pipetted onto nitrocellulose strips and incubated with ANA-P to test for esterase activity. Approximately 25 hemagglutinin units of virus were contained in each sample.

DISCUSSION

Influenza type C virus expresses an enzyme that destroys its specific host cell receptors, an activity analogous to the neuraminidase activity of influenza type A and B viruses. The enzyme activity of type C virus, unlike that of type A and B viruses, is inhibited by diisopropyl fluorophosphate, demonstrating that the enzyme is a serine esterase (15). Serine esterases are widely distributed in nature and hydrolyze a broad range of substrates (2, 24).

Influenza C virus is the only viral pathogen of the human upper respiratory tract presently known to produce an esterase. Detection of such activity facilitates the distinction of type C virus from type A and B influenza viruses. The uniqueness of the influenza C virus enzyme suggests an application of the esterase assay to distinguish type C virus from other respiratory viruses; this is supported by the lack of reactivity of various parainfluenza virus isolates with ANA-P.

The lack of endogenous esterase that is reactive with ANA-P in MDCK cells has provided a convenient system for studying the expression of the influenza C virus esterase in infected cells. Likewise, the lack of detectable amounts of ANA-reactive esterase in uninfected CAF and amniotic fluid provides a system to screen infected egg fluids for influenza type C virus. Reactivity of monkey kidney cells with ANA-P emphasizes the need for further study to find a substrate that may be used to detect influenza C virus in a wider variety of cell culture systems.

Herrler et al. (7, 8) reported that the influenza C virus esterase is reactive with 9-*O*-acetylneuraminic acid and that this is the receptor on erythrocytes. Hydrolysis of ANA and ANP by the influenza C esterase suggests a wider range of substrate specificity than previously reported. Our findings also support earlier work of Vlasak et al. (22), who showed that PNPA is a substrate for this enzyme.

Our experiments have shown that the time course of influenza C virus enzyme activity correlates with the production of infectious virus particles (17), beginning at approximately 8 h postinfection and with maximal activity at approximately 24 h. After 48 h, monolayers grown without an agarose-trypsin overlay show reduced esterase activity, indicating that production of virus is greatly decreased at this time.

The positive esterase assay of infected MDCK cells incubated without added trypsin suggests that enzymatic cleavage of the surface hemagglutinin glycoprotein, although necessary for fusion and infectivity of virus, is not necessary for esterase activity (19). This is apparent because MDCK cells lack the protease activity required to cleave the hemagglutinin and yet the infected cells have potent esterase activity in the absence of added trypsin.

Esterase assays of infected MDCK monolayers are clearly specific for influenza C virus and yield a negative test with type A and B viruses. Monolayers infected with influenza A or B virus showed no evidence of esterase activity, regardless of temperature, time of incubation, or type of medium used (liquid or agarose overlay). Further proof of the specificity of this assay was obtained by neutralizing influenza C virus with specific antisera, by incubating infected cells at nonpermissive temperatures, and by screening a variety of viral upper respiratory pathogens after application to nitrocellulose strips.

The sensitivity of the esterase assay permits detection of infected cells that might be overlooked with routine isolation procedures. The assay is sufficiently sensitive to detect esterase activity within 24 h postinfection, well before most plaques may be demonstrated. Furthermore, the esterase assay detects cell infections that do not mature into lytic plaques and thus may be more sensitive than the plaque assay. The esterase assay may be able to detect strains of virus that fail to produce lytic plaques in MDCK cells.

The detection of virus in fluids with PNPA as the substrate was not reliable, because this substrate undergoes autohydrolysis at alkaline pH. In addition, PNPA does not allow distinction of infected cells from adjacent uninfected cells in tissue culture.

We have found that screening of fluids from influenza C virus-infected eggs is easily accomplished by applying the fluids to nitrocellulose and incubating in ANA-P. Normal egg or tissue culture fluids or fluids containing influenza A or B or parainfluenza virus are very weakly and slowly reactive compared with fluids infected with any of the three strains of influenza C virus tested in our laboratory. This substrate also allows distinction in tissue culture between infected and uninfected cells.

The utilization of in situ esterase assays, whether in tissue culture or for assaying egg fluids, provides an easy means of detecting influenza C virus. The tests are quick, inexpensive, and easy to interpret and may distinguish influenza C virus from other influenza viruses. Previously, epidemiological studies of influenza C virus infections depended on serological analysis. A rapid and easy test to detect the virus in eggs or tissue culture should facilitate diagnosis of influenza C virus infections in infants and the elderly and may identify infections that presently are not recognized as being caused by this seldom-identified virus.

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LITERATURE CITED

1. **Andrewes, B. E., and J. C. McDonald.** 1955. Influenza C virus infection in England. Br. Med. J. **35**:992–997.
2. **Coates, P. M., M. A. Mestriner, and D. A. Hopkinson.** 1975. A preliminary genetic interpretation of the esterase isozymes of human tissues. Ann. Hum. Genet. **39**:1–20.
3. **Dykes, A., J. Cherry, and C. Nolan.** 1980. A clinical, epidemiologic, serologic, and virologic study of influenza C virus infection. Arch. Intern. Med. **140**:1295–1298.
4. **Francis, T., J. J. Quilligan, and E. Minuse.** 1950. Identification of another epidemic respiratory disease. Science **112**:495–497.
5. **Glezen, W. P.** 1980. Influenza C virus infections. Arch. Intern. Med. **140**:1278.
6. **Grist, N. R.** 1955. Influenza A and C in Glasgow, 1954. Br. Med. J. **35**:994–997.
7. **Herrler, G., and H.-D. Klenk.** 1987. The surface receptor is a major determinant of the cell tropism of influenza C virus. Virology **159**:102–108.
8. **Herrler, G., R. Rott, H.-D. Klenk, H.-P. Muller, A. K. Shukla, and R. Schauer.** 1985. The receptor-destroying enzyme of influenza C virus is neuraminate-*O*-acetylesterase. EMBO J. **4**:1503–1506.
9. **Homma, M.** 1986. Epidemiological characteristics of type C influenza, p. 125–138. *In* A. P. Kendal and P. A. Patriarca (ed.), Options for the control of influenza. Alan R. Liss, Inc., New York.
10. **Hornsleth, A., J. Siggaard-Andersen, and L. Hjort.** 1975. Epidemiology of herpesvirus and respiratory virus infections. I. Serologic findings. Geriatrics **45**:61–68.
11. **Jennings, R.** 1968. Respiratory viruses in Jamaica: a virologic and serologic study. III. Hemagglutination-inhibiting antibodies to type B and C influenza viruses in the sera of Jamaicans. Am. J. Epidemiol. **87**:440–446.
12. **Katagiri, S., A. Ohizumi, and M. Homma.** 1983. An outbreak of type C influenza in a children's home. J. Infect. Dis. **148**:51–56.
13. **Li, C. Y., K. W. Lam, and L. T. Yam.** 1973. Esterases in human leukocytes. J. Histochem. Cytochem. **21**:1–12.
14. **Minuse, E., J. J. Quilligan, and T. Francis.** 1954. Type C influenza virus. I. Studies of the virus and its distribution. J. Lab. Clin. Med. **43**:31–42.
15. **Muchmore, E. A., and A. Varki.** 1987. Selective inactivation of influenza C esterase: a probe for detecting 9-O-acetylated sialic acids. Science **236**:1293–1295.
16. **Nakada, S., R. Creager, M. Krystal, R. Aaronson, and P. Palese.** 1984. Influenza C virus hemagglutinin: comparison with influenza A and B virus hemagglutinins. J. Virol. **50**:118–124.
17. **Nerome, K., and M. Ishida.** 1978. The multiplication of an influenza C virus in an established line of canine kidney (MDCK) cells. J. Gen. Virol. **39**:179–181.
18. **O'Callaghan, R. J., R. S. Gohd, and D. D. Labat.** 1980. Human antibody to influenza C virus: its age-related distribution and distinction from receptor analogs. Infect. Immun. **30**:500–505.
19. **O'Callaghan, R. J., Sr. M. Loughlin, D. D. Labat, and C. Howe.** 1977. Properties of influenza C virus grown in cell culture. J. Virol. **24**:875–882.
20. **Rogers, G., G. Herrler, J. Paulson, and H. Klenk.** 1986. Influenza C virus uses 9-*O*-acetyl-*N*-acetylneuraminic acid as a high affinity receptor determinant for attachment to cells. J. Biol. Chem. **261**:5947–5951.
21. **Spence, H. A., and R. J. O'Callaghan.** 1985. Induction of chick embryo feather malformations by an influenza C virus. Teratology **32**:57–64.
22. **Vlasak, R., M. Krystal, M. Nacht, and P. Palese.** 1987. The influenza C virus glycoprotein (HE) exhibits receptor-binding (hemagglutinin) and receptor-destroying (esterase) activities. Virology **160**:419–425.
23. **Wenner, H., C. Christodoulopoulous, J. Weston, V. Tucker, and C. Liu.** 1963. The etiology of respiratory illnesses occurring in infancy and childhood. Pediatrics **31**:4–17.
24. **Yam, L. T., C. Y. Li, and W. H. Crosby.** 1971. Cytochemical identification of monocytes and granulocytes. Am. J. Clin. Pathol. **55**:283–290.